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Recombinant allergens in mould allergy
Investigations of the in vivo reactivity using a panel of recombinant allergens of Alternaria alternata and Cladosporium herbarum

INAUGURAL - DISSERTATION

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1 Summary

Atopic diseases as hav fever, allergic asthma and atopic dermatitis are of increasing prevalence. It is estimated that moulds account for around 20% of atopic diseases in atopic individuals and up to 6% in the general population. Cladosporium herbarum (*Cla h*), together with Alternaria alternata (*Alt a*) are two of the most important allergenic moulds. Diagnosis of atopic diseases is based on clinical history, skin tests, detection of specific serum IgE and provocation tests with the suspected allergen. Usually, extracts purified from raw material are used for these tests. Due to the included mixture from proteins and various extraction procedures, often only a limited standardization and purity is achievable. Recombinant allergens were found to be a very specific, reliable and safe tool for the diagnosis of mould allergic diseases and able to elicit allergic reactions in vivo.

In our study we investigated in a clinical trial of 29 mould allergic patients the use of selected recombinant mould allergens in skin prick tests (SPT), in comparison with natural mould allergen extracts. SPT were performed with recombinant allergens of *Cla h* and *Alt a* (*rAltNTF, rClaNTF, rAltMtDH, rClaMtDH, rAlta1, rClaTcTPEn*) and with natural allergen extracts of the two moulds. Skin tests with the recombinant allergen *rAlta1* were highly specific and detected more patients than skin tests with the allergen extracts. We were able to confirm that *Alta1* is a major allergen of *Alt a*. In spite of the large number of allergens in *Alt a*, it is likely that a combination of *Alta1* with a small number of other recombinant allergens could be sufficient to correctly diagnose *Alt a* sensitised patients with a high sensitivity and specificity. The other recombinant allergens that were investigated in our study, showed only in a very limited number of patients positive SPT and seem to be of lower importance. None

of the 17 controls showed a positive SPT. There were no side-effects recorded relating to the use of recombinant allergens in patients and controls.

Ongoing and future studies will have to determine the use of other recombinant allergens and also the therapeutic use of recombinant allergens for the specific immunotherapy of allergic diseases. However our findings strongly support the use of recombinant proteins in the diagnostic work up, especially for *Alt a* - related allergies and for the in vivo use.

Abbreviations

Alt a	Alternaria alternata
Cla h	Cladosporium herbarum
MtDH	Mannitol dehydrogenase
NTF2	Nuclear transport factor 2
r	Recombinant
RCA	Allergic rhinoconjunctivitis
SPT	Skin prick test

2 Introduction

2.1 Overview

Allergy is a hypersensitive response of the immune system to inhaled or ingested proteins, the allergens. In the past decades an increased prevalence of allergic diseases has been reported (1). Up to 20% of the population of the industrialized world are affected. Allergens constitute proteins from animals and plant sources (2, 3). Exposure to allergens results in the induction of IgE antibody, which is thought to be the central mechanism in the induction of an allergic reaction of the so-called immediate type. Allergic (extrinsic) asthma, allergic rhinitis, food allergy, drug allergy, insect sting reactions and to some extent atopic dermatitis and urticaria are classical IgE-associated allergies. The knowledge of a causing allergen often allows the avoidance of an allergen contact and has therefore a strong preventive character. The diagnosis of allergic diseases is based on a profound and well-structured clinical history and the detection of allergen-specific IgE results. The classical methods therefore are skin tests, detection of specific IgE in the serum and challenge of the affected organ by so-called provocation test.

Fungal allergy constitutes 25% to 30% of all allergic asthma cases (4, 5). Among the outdoor allergens, fungal spores, together with tree and grass pollen constitute major representatives. Furthermore, fungal spores are besides proteins from dust mite, cockroach and animal dander important indoor allergens as well. The prevalence rate of fungal allergy is estimated to be around 20% in atopic individuals and up to 6% in the general population (6-8). Attempts to establish the exact prevalence rate of fungal sensitation showed a vast variability what is ascribed to differences in the studied population, the tested species of fungi and the fungal extracts used (7).

Tens of thousands of fungal species are described currently. Until now, more than 80 genera of fungi have been associated with respiratory allergy, including yeasts and moulds (8). Fungi are eukaryotic, unicellular to multicellular, usually spore-bearing organisms. The term mould is often used synonymously with the term fungi, but actually describes a subgroup of mycelial fungi. Moulds occur ubiquitously in all continents and are present in all types of environment. The concentration of fungal spores in the environment depends on many factors, including climate and vegetation (9). Mould spores are present in outdoor air and inside the house, preferentially in bathrooms and basements, as well as in new habitations, in particular in case of exaggerated isolation and bad aeration of the room. Indoor spores constitute either form outdoor air or from moulds growing indoors. The types of moulds that are growing indoors and their prevalence depend on moisture, ventilation, presence or absence of carpets, pets, and houseplants (10).

Exposure to allergenic moulds may lead to IgE-mediated rhinitis and asthma, atopic dermatitis, or very rarely to systemic reactions such as urticaria or anaphylactic shock. Clinically, the presenting symptoms are sneezing, nasal discharge, coughing, wheezing, and shortness of breath, with evidence of reversible airway obstruction, rarely assocaited with other symptoms such as urticaria, angioedema, and even anaphylaxis.

2.2 Cladosporium herbarum and Alternaria alternata

Cladosporium herbarum (*Cla h*), together with Alternaria alternata (*Alt a*) are two of the most important allergenic moulds. A European multicenter study showed that the prevalence of *Cla h* and *Alt a* sensitisation varies widely in the different climatic zones of the world. It was demonstrated that 3 up to 20% of all allergic patients tested, showed positive responses to *Cla h* and / or *Alt a* in skin prick tests (11). Sensitised

patients were in most cases tested positive to several moulds. Monosensitization to just one mould species is found rarely. Whereas *Cla h* dominates in cooler climates, *Alt a* grows preferentially in warm and humid environments.

2.3 Diagnostics in mould allergy and recombinant allergens

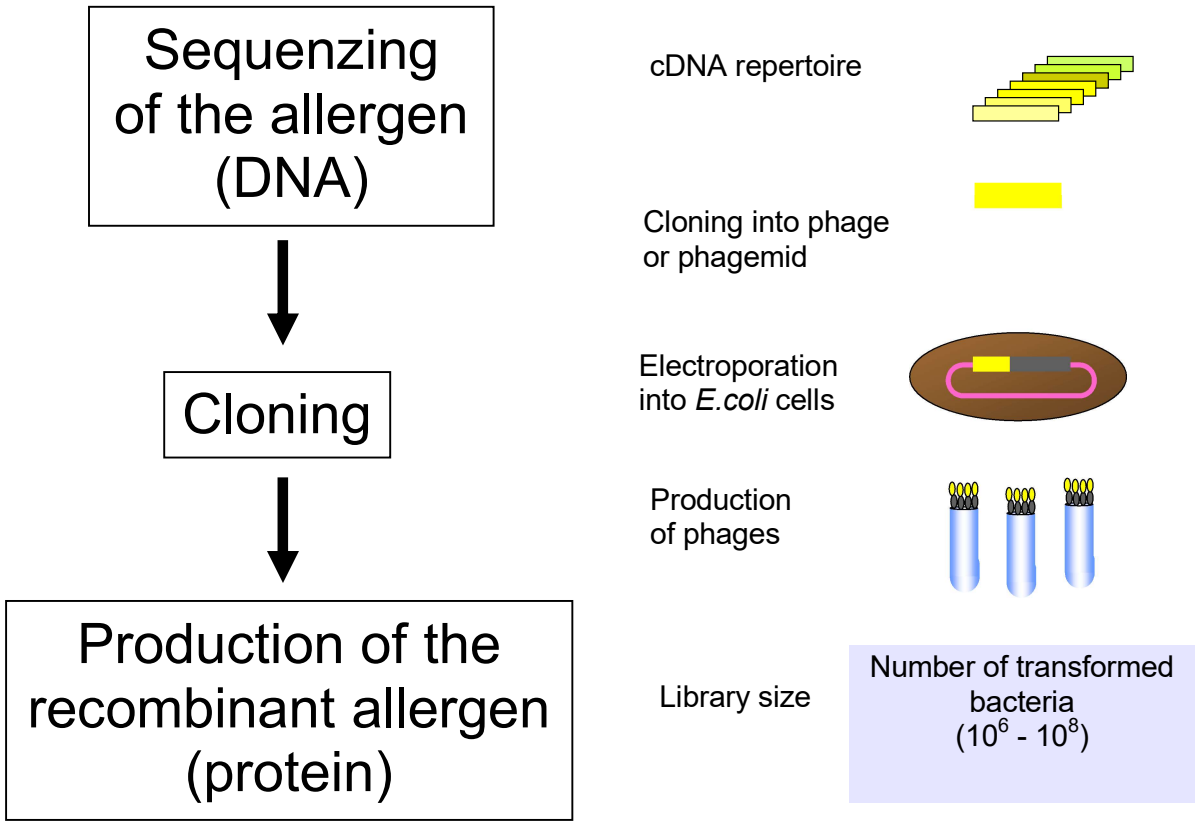
Besides clinical history, diagnosis of allergy is based on skin tests, provocation tests and in vitro investigations for determination of specific IgE. Skin tests are next to clinical history the basic tool in each allergological work-up. They are easily to perform and do allow a visible result within a few minutes. The in vitro tests most often used to determine specific serum IgE are radioallergosorbent test (RAST) (12), cellulose-absorbent paper test (CAP) (13, 14), and enzyme-linked immunosorbent test (ELISA). ImmunoCAPs are the most widely used method for the determination of allergen-specific IgE. In this test, allergens are immobilized onto cellulose solid phase by covalent binding. The coupling concentration is usually adjusted to attain a linear measurement range and a background below 0.35kU/l. Specific IgE in the serum is bound on allergen disks and is detected by colorimetry.

For skin tests, provocation tests and in vitro investigations, natural allergen extracts are used. These extracts are complex mixtures of relevant allergenic proteins, but contain also many non-allergenic substances, including other proteins and carbohydrates (15). Mould allergens are usually proteins, polysaccharides, or glycoproteins (16). Most allergen preparations derived from complex allergenic sources like moulds are still not yet available as internationally recognized, standardized products. Extracts derived from natural sources may vary in allergen composition and content. This was demonstrated in studies that showed a low correlation between skin test reactivity and reactivity of specific serum IgE using natural extracts (17). It has been difficult to obtain adequate quantities of fungal

extracts that are qualitatively and quantitatively reproducible in terms of allergen content. Furthermore there is variability in allergen content and in the amount of allergen produced from fungal sources because the quality of the extracts is influenced by many factors, including quality of the starting raw material, extraction procedure, protease content and storage conditions (18). Moreover, natural allergen extracts can be contaminated with allergens from other sources (19). Standardization of allergen extracts is therefore essential to avoid variation in sensitivity and specificity of allergy diagnoses (20).

The complexity and the variability of the fungal allergens have complicated the process of obtaining purified and standardized antigens. Considerable research has been directed to purify relevant antigens from fungi, but none of the applied methods has yielded relevant allergens useful in the diagnoses of mould allergy. However, recently, using molecular biology to clone and express relevant antigens from moulds, a possibility for standardization has developed (21). The resulting recombinant allergens represent highly pure protein preparations free of contaminants deriving from natural allergenic sources. Many allergens have been cloned, and the proteins expressed. Currently, about 100 fungal allergens have been approved by the International Allergen Nomenclature Committee (22). The cloning of allergens consists of the isolation and purification of messenger RNA from actively growing cultures (23). The purified messenger RNA is subsequently reverse transcribed to obtain cDNA and finally the desired DNA fragments are cloned and amplificated by polymerase chain reaction (PCR). The expression of the protein is induced in bacterial host, usually in Escherichia coli (*Figure 1*).

Figure 1: Production of recombinant allergens in E. coli



Although cloning and characterization of single allergens may be time consuming, the advantages are manifold. Recombinant allergens can be carefully quantified in terms of protein concentration, and IgE reactivity would not be interfered by irrelevant proteins, macromolecules, or enzymes (24, 25). Therefore, recombinant allergens can be used as a 'golden standard' for both in vitro and in vivo tests, allowing objective comparisons of test results obtained in different studies and with different methods. Furthermore, recombinant allergens are powerful reagents to study the mechanisms involved in allergic reactions (26) and to study structure-function relationships (27).

Skin testing with recombinant allergens offers a highly specific and safe diagnostic tool to elucidate patient- and disease-specific sensitization patterns. Mostly due to

ethical and legal restrictions regulating the use of recombinantly produced proteins in humans, skin tests with recombinant Allergens have been rarely performed. The first skin tests with recombinant Allergens have been described in 1992 (28), many years after cloning of the first allergen from house dust mite (29). Recently, more and more work has been done in this direction and recombinant allergens pollen, mites, different fungi, bee venom, various foods and latex have been evaluated in skin tests as reviewed recently (30).

2.4 Aims of the study

The aim was to contribute more data about the diagnostic reliability of recombinant allergens for clinical applications.

The goal was to evaluate the specificity, sensitivity and safety of selected recombinant mould allergens in comparison with natural mould allergen extracts by skin testing.

The patients in our study were sensitised to natural allergen extracts of *Cla h* and / or *Alt a*. As mentioned earlier, moulds are complex allergenic sources that contain large amounts of IgE-binding molecules, covering many different species-specific and cross-reactive structures. Several IgE-binding structures of *Cla h* and *Alt a* have been cloned and characterized to date (30-38). We utilized the following recombinant allergens:

<u>rAlta1</u>

This 30kD Protein reacts with IgE of the majority of *Alt a* - sensitized patients. It is a secreted protein, which presumably resides in the periplasmatic space of *Alt a* cells. Up to now, extensive homology searches revealed no *Alta1* homolog from other species and thus there is no clue to the biological function of this major allergen (32, 36).

rAltNTF / rClaNTF

Nuclear Transport Factor 2 (*NTF2*) is a protein with a molecular weight of approximately 15kD. It is essential for maintaining cellular nuclear transport and cell viability. *NTF2* represents a cross-reactive fungal allergen. *Alt a* and *Cla h* show a sequence identity of 64% (35).

r Alt MtDH / r Cla MtDH

Mannitol dehydrogenase is a recently cloned, 28kD Protein (37, 38). It is a NADP-dependent, cytoplasmatic protein which is abundant in vegetative cells of moulds. It has been shown that mannitol dehydrogenase is a stress-inducible protein (39, 40). Its physiological importance may be seen in the interaction between the mould and its environment. There is evidence that mannitol dehydrogenase is a first major allergen of *Cla h* (37) and the second most important allergen of *Alt a* that has been identified so far (38). Cross-inhibition ELISA showed that *MtDH* is cross-reactive between *Alt a* and *Cla h* and that in addition to shared IgE-epitopes, there also exist mould-specific epitopes as no complete cross-inhibition could be achieved (38).

r Cla TcTPEn

The translationally controlled tumor-associated proteins (*TCTPs*) are a highly conserved and abundantly expressed family of eukaryotic proteins that are implicated in both cell growth and the human acute allergic response but whose intracellular biochemical function has remained elusive (41).

3 Patients, Materials and Methods

3.1 Patients and controls

Three different groups of participants were included into the study.

- Group 1: mould allergic patients (29 pts)
- Group 2: allergic patients sensitized to inhalant allergens but not to moulds (6 pts)
- Group 3: healthy, non-allergic controls (11 individuals)

Group 1 included 29 patients with typical symptoms of inhalant allergies and sensitation against moulds. Diagnosis of mould allergy was based on elevated specific IgE serum levels to *Cla h* and / or *Alt a*, determined by CAP-RAST test results (ImmunoCAP Pharmacia) together with a clinical history of recurrent rhinitis (possibly also additional conjunctivitis and / or asthma). All patients showed stable lung functions.

Group 2 included 6 patients with a history of allergic symptoms such as saisonal rhinoconjuncitivitis and asthma. All showed sensitizations against common inhalant allergens such as mites, pollen or animal dander but not to moulds, which was shown by a negative screening test for fungal allergens (Mx1).

Group 3 included 11 healthy, non-allergic controls. Individuals were defined by the absence of a history of allergic symptoms and not detectable specific IgE against common inhalant, food and fungal allergens (negative screening tests by CAP RAST for Sx1, Mx1, Fx5).

Characteristics of patients and controls are displayed in *tables 1 and 2*.

Table 1: Characteristics of patients

number of patients included	29
mean age (range)	35 (16-61)
gender m/f	15/14
CAP m2 (>0.5 kU/l)	17/29 (59%)
CAP m6 (>0.5 kU/l)	26/29 (90%)

Table 2: Characteristics of controls

	allergic controls	healthy controls
number of controls included	6	11
mean age (range)	31 (23-57)	32 (26-62)
gender m/f	4/2	5/6
Sx1 (<0.35 kU/l)	0/7 (0%)	11/11 (100%)
Mx1 (<0.35 kU/l)	7/7 (100%)	11/11 (100%)
Fx5 (<0.35 kU/l)	7/7 (100%)	11/11 (100%)

The patients were recruited from our allergy unit on the basis of positive CAP-RAST test results which where obtained during the years 2000-2004.

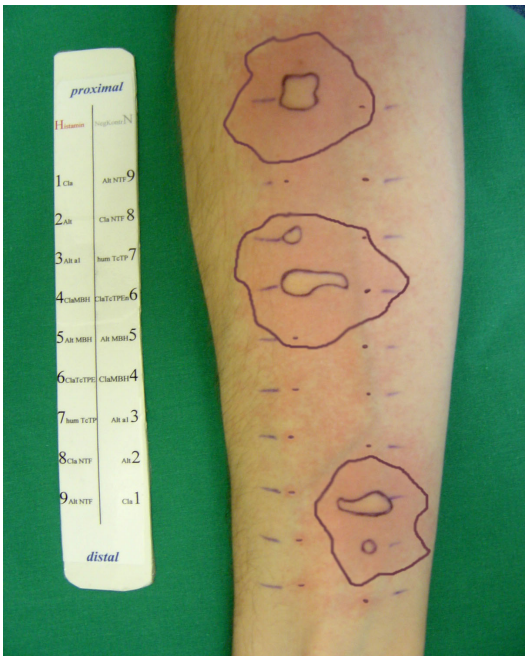
During the study, patients were not allowed to use either antihistamines or topical or systemic steroids, starting at least one week before the investigations.

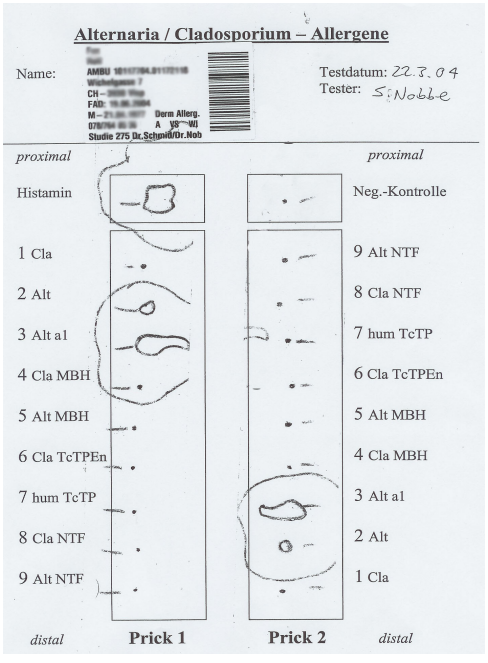
The study was approved by the Ethical Committee of the University of Zurich. A full oral and written explanation of the procedure was given to the patients and controls. All patients and controls gave oral and written informed consent before testing.

3.2 SPT with extracts and recombinant allergens

SPT were performed with commercial extracts and recombinant allergens from *Cla h* and *Alt a* (ALK Amersham, Denmark). Sodium chloride (0.9%) and histamine hydrochloride (ALK Amersham, Denmark) served as negative and positive controls respectively. All SPTs were performed twice on the volar forearm and applied in the opposite directions. Twenty microliters of the allergen solutions were used per test. SPTs were read after 20 min. Wheals and flares were penmarked and then transferred with scotch tape to a paper. Practical realisation of SPT and documentation is displayed on *figure 2*. SPT was considered positive when a mean diameter of ≥ 4 mm was achieved. Only patients with a SPT to negative control with 0.9% sodium chloride with a wheal diameter below 1 mm were included in the study.

Figure 2: practical realisation of SPT and documentation





3.3 Determination of allergen-specific IgE

From all patients and controls, specific IgE to natural extracts of *Cla h* and *Alt a* were determined by the Immuno-CAP System. Results are displayed in table 3a.

4 Results

4.1 SPT with extracts and recombinant allergens

Of 29 patients with mould-allergy, 25 patients showed positive skin test reactions (86%). 4 patients showed no reactions to mould allergens (14%). Of the 25 patients with positive skin test reactions, 22 patients reacted either to allergens of *Cla* h or to allergens of *Alt a* (7 patients and 15 patients respectively). 3 patients showed positive skin tests to allergens of both of the moulds. Positive skin tests with *Cla* h allergens were achieved with commercial extracts (10 patients), with *rClaMtDH* (1 patient) and with *rClaNTF* (1 patient). We found no positive reactions with *rClaTcTPE*n.

Positive skin tests with *Alt a* allergens were achieved with commercial extracts (13 patients), with *rAlta1* allergen (17 patients), and with *rAltNTF* (1 patient). No positive results were found with *rAltMtDH*.

In the control groups, no positive skin tests with commercial mould extracts or recombinant allergens were achieved. To mention is that a primarily in the healthy control group assigned person showed a clearly positive skin prick reaction (8mm) to *rAlta1* allergen. This person negated any allergic symptoms but was excluded from the healthy control group because screening CAP-RAST Tests indicated sensitation to several antigens (Sx1 0.71kU/l, Mx1 0.37kU/l, m6 0.42kU/l).

All results are displayed in *tables 3a and 3b*.

Table 3a: In vivo and in vitro pattern of sensitization to fungal Allergens

Nr.	History	SPT Cla (mm)	SPT Alt (mm)	SPT rAlta1 (mm)	other SPT (mm)	CAPm2 (kU/l)	CAPm2 (kU/l)
1	RCA	negative	4.0	10.5	negative	3.30	27.40
2	RCA	negative	4.0	9.0	negative	1.59	15.60
3	RCA	4.0	negative	negative	negative	0.47	1.33
4	RCA	4.0	5.0	12.0	negative	11.40	27.20
5	RCA	7.0	negative	negative	negative	9.63	<0.35
6	RCA	negative	negative	negative	negative	16.80	49.20
7	RCA	7.0	negative	negative	negative	13.10	<0.35
8	RCA	5.0	negative	negative	negative	6.08	<0.35
9	RCA	negative	negative	7.0	negative	1.19	12.60
10	RCA	negative	negative	negative	negative	0.83	0.72
11	RCA	negative	negative	negative	negative	1.04	2.20
12	RCA	negative	negative	10.5	negative	1.73	43.70
13	RCA	7.5	negative	negative	rClaMtDH 11.5	29.50	0.96
14	RCA	7.0	8.0	10.0	negative	<0.35	4.40
15	RCA	negative	negative	negative	negative	<0.35	2.45
16	RCA	negative	5.0	12.5	negative	<0.35	1.26
17	RCA	negative	4.0	10.5	negative	<0.35	4.45
18	RCA	negative	4.0	12.0	negative	1.77	18.90
19	RCA	negative	5.0	10.0	negative	<0.35	3.97
20	RCA	negative	5.5	9.5	negative	2.44	18.10
21	RCA	negative	4.5	12.5	negative	<0.35	6.14
22	RCA	5.0	negative	negative	negative	13.10	0.53
23	RCA	4.0	5.0	8.5	negative	1.01	10.80
24	RCA	negative	negative	11.0	negative	<0.35	10.20
25	RCA	negative	5.0	10.0	negative	<0.35	27.50
26	RCA	negative	negative	10.5	negative	<0.35	1.04
27	RCA	4.0	negative	negative	negative	1.36	1.89
28	RCA	negative	negative	8.5	negative	<0.35	1.55
29	RCA	negative	5.0	negative	rCla/AltNTF4.0	<0.35	0.66
30	healthy	negative	negative	negative	negative	<0.35	<0.35
31	healthy	negative	negative	negative	negative	<0.35	<0.35
32	healthy	negative	negative	negative	negative	<0.35	<0.35
33	healthy	negative	negative	negative	negative	<0.35	<0.35
34	healthy	negative	negative	negative	negative	<0.35	<0.35
35	healthy	negative	negative	negative	negative	<0.35	<0.35
36	healthy	negative	negative	negative	negative	<0.35	<0.35
37	healthy	negative	negative	negative	negative	<0.35	<0.35
38	healthy	negative	negative	negative	negative	<0.35	<0.35
39	healthy	negative	negative	negative	negative	<0.35	<0.35
40	healthy	negative	negative	negative	negative	<0.35	<0.35
41	RCA	negative	negative	negative	negative	<0.35	<0.35
42	RCA	negative	negative	negative	negative	<0.35	<0.35
43	RCA	negative	negative	negative	negative	<0.35	<0.35
44	RCA	negative	negative	negative	negative	<0.35	<0.35
45	RCA	negative	negative	negative	negative	<0.35	<0.35
46	RCA	negative	negative	negative	negative	<0.35	<0.35

Table 3b: In vivo pattern of sensitization to recombinant fungal allergens

Nr.	SPT Cla (mm)	SPT Alt (mm)	SPT rAlta1 (mm)	SPT rAltNTF (mm)	SPT rClaNTF (mm)	SPT rAltMtDH (mm)	SPT rClaMtDH (mm)	SPT rClaTcTPEn (mm)	SPT humTcTp (mm)
1	neg	4.0	10.5	neg	neg	neg	neg	neg	neg
2	neg	4.0	9.0	neg	neg	neg	neg	neg	neg
3	4.0	neg	neg	neg	neg	neg	neg	neg	neg
4	4.0	5.0	12.0	neg	neg	neg	neg	neg	neg
5	7.0	neg	neg	neg	neg	neg	neg	neg	neg
6	neg	neg	neg	neg	neg	neg	neg	neg	neg
7	7.0	neg	neg	neg	neg	neg	neg	neg	neg
8	5.0	neg	neg	neg	neg	neg	neg	neg	neg
9	neg	neg	7.0	neg	neg	neg	neg	neg	neg
10	neg	neg	neg	neg	neg	neg	neg	neg	neg
11	neg	neg	neg	neg	neg	neg	neg	neg	neg
12	neg	neg	10.5	neg	neg	neg	neg	neg	neg
13	7.5	neg	neg	neg	neg	neg	11.5	neg	neg
14	7.0	8.0	10.0	neg	neg	neg	neg	neg	neg
15	neg	neg	neg	neg	neg	neg	neg	neg	neg
16	neg	5.0	12.5	neg	neg	neg	neg	neg	neg
17	neg	4.0	10.5	neg	neg	neg	neg	neg	neg
18	neg	4.0	12.0	neg	neg	neg	neg	neg	neg
19	neg	5.0	10.0	neg	neg	neg	neg	neg	neg
20	neg	5.5	9.5	neg	neg	neg	neg	neg	neg
21	neg	4.5	12.5	neg	neg	neg	neg	neg	neg
22	5.0	neg	neg	neg	neg	neg	neg	neg	neg
23	4.0	5.0	8.5	neg	neg	neg	neg	neg	neg
24	neg	neg	11.0	neg	neg	neg	neg	neg	neg
25	neg	5.0	10.0	neg	neg	neg	neg	neg	neg
26	neg	neg	10.5	neg	neg	neg	neg	neg	neg
27	4.0	neg	neg	neg	neg	neg	neg	neg	neg
28	neg	neg	8.5	neg	neg	neg	neg	neg	neg
29	neg	5.0	neg	4.0	4.0	neg	neg	neg	neg
30	neg	neg	neg	neg	neg	neg	neg	neg	neg
31	neg	neg	neg	neg	neg	neg	neg	neg	neg
32	neg	neg	neg	neg	neg	neg	neg	neg	neg
33	neg	neg	neg	neg	neg	neg	neg	neg	neg
34	neg	neg	neg	neg	neg	neg	neg	neg	neg
35	neg	neg	neg	neg	neg	neg	neg	neg	neg
36	neg	neg	neg	neg	neg	neg	neg	neg	neg
37	neg	neg	neg	neg	neg	neg	neg	neg	neg
38	neg	neg	neg	neg	neg	neg	neg	neg	neg
39	neg	neg	neg	neg	neg	neg	neg	neg	neg
40	neg	neg	neg	neg	neg	neg	neg	neg	neg
41	neg	neg	neg	neg	neg	neg	neg	neg	neg
42	neg	neg	neg	neg	neg	neg	neg	neg	neg
43	neg	neg	neg	neg	neg	neg	neg	neg	neg
44	neg	neg	neg	neg	neg	neg	neg	neg	neg
45	neg	neg	neg	neg	neg	neg	neg	neg	neg
46	neg	neg	neg	neg	neg	neg	neg	neg	neg

4.2 Determination of allergen-specific IgE

The results performed with the Immuno-CAP System are displayed in table 3a. As being one of the inclusion criteria, patients had either elevated IgE-levels for *Cla h* or *Alt a*.

5 Discussion

Mould allergy is a not very frequent but nevertheless clinically relevant problem in about 3 to 6% of the population. However, diagnosis of fungal allergy remains a difficult task. The complexity and the variability of the fungal allergens don't allow to obtaining purified and well-standardized extracts. Considerable efforts have been directed to purify relevant antigens from fungi, but none of the applied methods has yielded relevant allergens useful in the diagnosis of mould allergy. However, recombinant allergens with well standardized and highly pure allergenic proteins are a possible tool to improve the diagnosis of mould allergies. The detection of in vitro IgE-binding with recombinant allergens not necessarily leads to allergenicity in vivo, due for example to incorrect folding or tertiary structure. Thus we wanted to investigate the in vivo reactivity of such single recombinant fungal allergens in mould allergic patients by performing skin prick tests.

We could examine only a relatively limited number of patients due to the fact that relevant sensitization against moulds is rather rarely detectable. We recruited our patients from the basis of positive CAP-RAST test results that were obtained during allergy screening tests. The limited number of recruitable patients is due to various reasons:

- in general, allergy to moulds affects only about 3-6% of the population compared to up to 18% of the population suffering from pollinosis
- the symptoms of mould allergy are often not very clear attributable to a certain season or place and therefore missed more often
- the sensitivity and specificity of the current diagnostic tools (Skin tests and specific IgE) are very low, so fungal allergy can be missed quiet easily

In our study we were able to confirm the importance of the major allergen *Alta1* in *Alt a* - sensitised patients.

More than 90% of the patients with positive skin tests to commercial extracts were tested positive to recombinant *Alta1* - allergen. According to the literature and now demonstrated by our findings, it seems to be by far the most important antigen of *Alt a* (32, 36). The other recombinant *Alt a* allergen that showed a positive SPT, *rAltNT*F, is likely to be less important, since only one single patient had a positive SPT. Thus we postulate that it is a minor allergen of *Alt a*. Surprisingly, we found no positive skin reactions with *rAltMtDH*. We expected positive results because recent in vitro investigations (IgE-ELISA and immunoblots) showed that *MtDH* is recognized by 41% of *Alt a* - allergic patients (38). Possible reasons for these divergent results are discussed later.

Interestingly, more serological *Alt a* - sensitized patients reacted in skin tests with the recombinant protein *Alta1* than with the commercial extract: 12 patients were tested positive both for *rAlta1* and extract, 5 patients reacted solely to *rAlta1* and only 1 patient reacted solely to the extract. There were no positive skin tests in patients or controls. The findings underline on one side the very high specificity of the recombinant allergens. On the other side, in the case of *Alt a* with a rather heterogenous commercial extract, and with *rAlta1* seeming to be the most important allergen, we found an even higher sensitivity of the recombinant allergen compared to the commercial extract.

In spite of the large number of allergens in *Alt a*, it is most likely that a combination of *rAlta1* with a small very limited number of other recombinant allergens of *Alt a* is sufficient to correctly diagnose sensitised patients with a sensitivity and a specificity superior to those obtained with the nowadays available commercial extracts.

The same has been shown by Asturias et al. in 41 patients using natural *Alta1* and two different forms of recombinant *Alta1* (42).

In other studies it has been found that the use of recombinant allergens for skin tests gives an excellent specificity which often reaches 100% (18). Knowing that different allergens of a species can be responsible to elicit allergy, it is understandable that the sensitivity of skin tests with single recombinant allergens is generally lower than those obtained with commercial extracts. An increasing numbers of recombinant allergens will therefore raise the sensitivity of the diagnosis. The minor allergen *rAltNTF* may contribute to reduce the number of structures required for such a diagnostic test (35).

Our results in the investigations with allergens of *Cla h* were not as meaningful as the results with allergens of *Alt a*. Positive SPTs were achieved in only one patient with *rClaNTF* and in another patient with *rClaMtD*H. Regarding the results of SPTs in our study, these two allergens seem not to be major allergens of *Cla h*. A major allergen like *Alta1* in *Alt a* has not been found until now. However, recently published investigations presented cloning, production and characterization of *ClaMtDH* stated that *ClaMtDH* is a major allergen of *Cla h*, since it could be shown that more than 50% of the Cla h allergic patients displayed specific IgE-reactivity with the pure *rClaMtDH* (37). The reason for the discrepancy between skin tests and in vitro results might be due to incorrect folding of the molecule or lack of glycosilation that does not occur in recombinant allergens produced in E. coli (43).

Assuming, *ClaMtDH* would be a major allergen of *Cla h*, it is likely that the combination of allergens that are sufficient to diagnose patients sensitised to *Cla h* has to be much larger than the combination of *Alt a* allergens, since the incidence of sensitization to *ClaMtDH* seems not to be as frequent as the incidence of sensitization to *Alta1*.

SPT with recombinant allergens proved to be safe as no adverse side effects, neither allergic nor toxic, have been observed in our study. This is also observed in more than 45 other studies, where no serious adverse effects due to skin tests with recombinant allergens occurred in more than 1400 patients tested (18).

To conclude, the major advantages of recombinant allergens compared to allergen extracts in the diagnosis of allergic diseases are:

- the possibility to produce highly standardized quantifiable reagents for diagnostic applications in vivo and in vitro
- an excellent specificity, reaching 100% in most of the published studies
- a good safety, as side effects are very rare due to the quantifiable amount of protein applied
- the possibility to investigate a single allergen instead of complex mixtures of allergens present in extracts, allowing insights into the epidemiologic and functional role of single molecules in allergic conditions

Another important use of recombinant allergens might be their applications in specific immunotherapy with the possibility to manipulate the coding sequences of allergens to produce hypoallergenic variants, e.g. molecules retaining the important structural features for the induction of T-cell-mediated immune responses but lacking the capability to bind IgE. Especially in patients with mould allergy, the possiblities of immunotherapy are still very disappointing (44-46). Mostly due to the lack of standardization of the now available extracts, the use of recombinant allergens may substantially contribute to a improvement of immunotherapy.

There are also disadvantages in the use of recombinant allergens:

- for complex allergenic sources like moulds, a panel of multiple recombinant allergens is needed to reach a sufficient sensitivity in the detection of patients with different sensitization patterns, limiting the use of recombinant allergens for routine skin tests
- the allergenicity of recombinant allergens in vivo is lower than that of natural allergens due to the fact that possibly not all of the relevant allergens are contained in the panel of recombinant allergens used
- the costs for the preparation of recombinant allergens are still substantially higher than for the production of allergen extracts. However this will change within the next years due to the important progress made in the industrialized production of recombinant proteins as e.g. already widely used for other drugs such as insulin.
- due to legal and ethical restrictions the wide use of recombinant allergens in vivo is still limited in many nations. All these proteins have to be synthesized under ‘good manufacturing conditions’ (GMP-like) which costs substantially and still limits the use of these proteins in clinical work and for research use.

In conclusion, our skin prick test study showed a high reliability for *Alta1* to detect *Alt a* - allergic patients. Sensitization against the other tested allergens seems to be either very low or the in vivo allergenicity of these proteins is reduced due to the manufacturing process. Further investigations and testing with recombinant mould allergens in large groups of preferential exclusive mould allergic patients are necessary to find out more about mould allergy and to discover more important allergens to finally profit from the benefits and advantages of recombinant allergens.

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